

## Using small molecule probes to investigate aggregation of sunset yellow FCF: what are the concentration limits?

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**Using Small Molecule Probes to Investigate Aggregation of  
Sunset Yellow FCF: What are the Concentration Limits?**

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**Using Small Molecule Probes to Investigate Aggregation of Sunset Yellow FCF:**  
**What are the Concentration Limits?**

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**Abstract**

The assembly of small molecules into larger structures, often driven by non-covalent interactions such as hydrogen-bonding, aromatic stacking interactions and burial of hydrophobic surface, is of wide spread interest. The interaction of small molecules with aggregates also has a large range of application from fluorescence aggregation assays to gas storage in framework materials. Here we utilise nuclear magnetic resonance spectroscopy to investigate the interaction of a small molecule probe on the assembly state of sunset yellow across a wide range of relative concentrations. Information from both macroscopic (diffusion) and microscopic (chemical shifts) measurements allows the interaction to be studied and the binding mode to be interrogated. Using fluorophenol as the small molecule probe, we show that the aggregation behaviour of sunset yellow is broadly unaffected by the relative amount of fluorophenol added.

## Introduction

The aggregation of small molecules into larger non-covalent assemblies has implications across a large number fields.<sup>1-4</sup> For example, the physicochemical properties of active pharmaceutical ingredients, such as apparent lipophilicity and the ability to permeate membranes, can be altered by aggregation, as can the results of biochemical assays in screening campaigns causing false positives and/or negatives.<sup>5-8</sup> The assembly of small proteins in larger assemblies is well known and actively studied in the context of various human pathologies including Alzheimer's and Parkinson's diseases.<sup>2,9</sup> Assemblies of small molecules, e.g. metal-organic frameworks, are also being utilised in a number of areas, including gas filtration and storage applications.<sup>10</sup>

Sunset yellow is an archetypical and well-studied example of a molecule which undergoes self-association in aqueous solution.<sup>1,3,11-15</sup> This association is driven predominantly by  $\pi$ - $\pi$  stacking interactions which seek to reduce the hydrophobic surface exposed to solvent.<sup>16,17</sup> At low concentration, sunset yellow is known to form long rod-like indefinite assemblies with a distribution of rod lengths.<sup>13,18</sup> As the concentration is increased, these rod can interact with each other, leading to the formation of lyotropic liquid crystalline phases above certain compositions or below the phase transition temperature.<sup>11-13</sup> A number of techniques have been employed to investigate the assembly of sunset yellow, both in isotropic and liquid crystalline phases, including scattering techniques,<sup>11-13</sup> nuclear magnetic resonance (NMR) spectroscopy,<sup>3,15,18,19</sup> and molecule dynamics simulations.<sup>20</sup> More recently, the stability of the hydrazone tautomer has been studied by NMR spectroscopy and DFT calculations.<sup>21</sup> This study demonstrates that the internal hydrogen bond is particularly

1  
2  
3 stable, with no exchange evident between the hydrogen atom and water trapped  
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5 within the aggregates.<sup>21</sup> Deuterium NMR has been employed to probe the dynamics  
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7 and ordering in mesophases formed by sunset yellow. Changes in the long range order  
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9 parameters appear to occur on timescales much slower than fast assembly of the  
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11 aggregates.<sup>22</sup>  
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17 The presence of other species in the sample is known to influence the assembly of  
18  
19 sunset yellow, particularly the addition of monovalent salts, such as sodium chloride,  
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21 leads to a stabilisation of the liquid crystalline phase<sup>14</sup> along with a change in the  
22  
23 reorientational dynamics as measured by proton relaxometry.<sup>23</sup> There is evidence that  
24  
25 the effect of added sodium chloride on the phase behaviour appears to be different at  
26  
27 high and low concentrations of sunset yellow.<sup>24,25</sup> The addition of crowding agents,  
28  
29 such as poly(ethylene glycol) has been reported to cause a separation of sunset yellow  
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31 samples into areas rich in liquid crystalline material and PEG-rich isotropic regions.<sup>24</sup>  
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37 The interaction of dye molecules with larger structures is of wide interest. Sunset  
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39 yellow interactions with amyloid fibril aggregates of lysozyme has been studied using  
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41 range of biophysical techniques, including transmission electron microscopy and  
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43 various fluorescence assays.<sup>26</sup> These investigations demonstrated that sunset yellow  
44  
45 binds to exposed hydrophobic surface such as solvent accessible tryptophan  
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47 residues.<sup>26</sup> Related to this, the binding of sunset yellow to pepsin has implications for  
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49 its use as a food additive. A combination of fluorescence and molecular dynamic  
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51 simulations reveals that sunset yellow causes an inhibition of pepsin, binding to a  
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53 cluster of tyrosine and serine residues in the active site.<sup>27</sup>  
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Previously, we have undertaken a series of NMR investigations of the aggregation state of sunset yellow as a function of concentration in the isotropic phase.<sup>18,28,29</sup> These studies have used both bulk parameters, such as the diffusion coefficient,<sup>18</sup> and the <sup>1</sup>H chemical shifts to examine the local environment within the aggregates.<sup>28,29</sup> The results concurred with existing literature data<sup>3,13,24</sup> in that there is a broad distribution of aggregate sizes present in solution, which tends towards extended, rod-like, structures at higher concentrations.<sup>18</sup> Following on from this, we have employed small molecule probes, possessing similar structural features to sunset yellow, as potential reporters on the aggregation processes.<sup>28,29</sup> These additional species were present at low relative concentration (1 mol%) with the aim of causing as little disturbance as possible to the overall assembly of the sunset yellow, while still being able to report on the aggregation state. Information from unique NMR reporters present on the added small molecule, such as <sup>19</sup>F (and <sup>31</sup>P, unpublished), via chemical shifts and diffusion coefficients allowed the characterisation of different binding modes of the small molecule probe, both to the ends and to the interiors of the assemblies.<sup>28–30</sup> A change-over in binding mode, from predominantly end-on to insertion, was observed as the concentration of sunset yellow increased, alongside the concomitant increase in the size of the sunset yellow aggregates present in solution.<sup>28</sup>

In this paper we extend our previous studies on the interaction of the three fluorophenol isomers with sunset yellow.<sup>28</sup> The effect of increasing the concentration of fluorophenol on the association behaviour of sunset yellow, within the isotropic phase, is investigated up to an equimolar mixture of probe and SSY aggregate. 3-fluorophenol is used as the exemplar, with similar results being obtained for the other structural isomers. The data is interpreted in terms of the simple isodesmic model of

indefinite self-association<sup>31</sup> as used previously.<sup>28,29</sup> Similarities and differences between the various isomers of probe molecule are noted in terms of the differences in polarity and degree of association.

## Materials and Methods

### *Sample preparation*

Sunset yellow FCF (**1**) and fluorophenol (**2**, **3** and **4**) were purchased from Sigma Aldrich (Dorset, UK). The structures are shown in Figure 1. D<sub>2</sub>O was from Goss Scientific (Cheshire, UK). Sunset yellow was purified by two rounds of ethanol precipitation, filtration and drying in a vacuum oven prior to use.<sup>13,14,18</sup> All other chemicals used as obtained. Samples were prepared via serial dilution from stock solutions of ~700 mM sunset yellow and 500 mM of the relevant fluorophenol isomer. Samples of >400 mM sunset yellow were prepared with up to 50 mol% fluorophenol due to solubility issues at higher fluorophenol mole fractions. The concentration of the sunset yellow stock solution was determined by UV/visible spectrophotometry using an extinction coefficient of 8270 M<sup>-1</sup> cm<sup>-1</sup> at 523 nm, as reported previously.<sup>28,29</sup> Samples were confirmed to be in the isotropic phase by the absence of any quadrupolar splitting in the <sup>2</sup>H NMR spectrum.<sup>18,28</sup>

### *NMR spectroscopy*

Spectra were recorded on a Varian VNMRS 600 (Yarnton, UK) using an X{<sup>1</sup>H-<sup>19</sup>F} broadband probe equipped with a z-gradient capable of 0.7 T m<sup>-1</sup>. The sample temperature was regulated at 298 K. Typical parameters for the <sup>1</sup>H and <sup>19</sup>F spectra were: 16k complex points over a spectral window of 9.6kHz, and 32k complex points over 28.4 kHz respectively. Spectra were acquired with between 16 and 128 transients



depending on the sample concentration. The resulting data were processed with the Mestrenova suite (Santiago de Compostela). Diffusion NMR experiments were performed using the Oneshot sequence<sup>32</sup> with typical parameters being 16 or 32 gradient points of between 1.5 and 3 ms duration ( $\delta$ ), equally spacing in  $g^2$  between 0.0452 and 0.5650 T m<sup>-1</sup>. The diffusion encoding time  $\Delta$  was 100 ms. Spectra were processed with 1 Hz exponential line broadening prior to Fourier transformation. Diffusion data were fitted to a monoexponential Stejskal-Tanner equation:

$$s(g) = s(0)\exp(-\gamma^2 g^2 \delta^2 D \Delta') \quad (1)$$

using DOSY Toolbox.<sup>33</sup>  $\Delta'$  is the diffusion delay suitably modified for the Oneshot sequence<sup>32</sup> and  $\gamma$  is the magnetogyric ratio.

#### *Diffusion coefficient analysis*

The measured diffusion coefficient of a species undergoing rapid two site exchange on the time scale of the diffusion labelling period  $\Delta$  is given by the Lindman law:<sup>34</sup>

$$D_{\text{obs}} = p_{\text{free}} D_{\text{free}} + p_{\text{bound}} D_{\text{bound}} \quad (2)$$

where  $p_{\text{free}} + p_{\text{bound}} = 1$ . In the context of the interaction of a small molecule with an aggregate, the observed diffusion coefficient of the reporter molecule (fluorophenol) is:<sup>28</sup>

$$D_{\text{nFP}} = \chi_{\text{free}} D_{\text{nFP, free}}^{\text{corr}} + \chi_{\text{asc}} D_{\text{SSY}} \quad (3)$$

where  $\chi_{\text{free}} + \chi_{\text{asc}} = 1$ . The diffusion coefficient of the probe molecule in free solution was corrected for changes in the microscopic viscosity due to the presence of the aggregated species as follows:

$$D_{\text{nFP, free}}^{\text{corr}} = \frac{D_{\text{HOD}}}{D_{\text{HOD}}^0} D_{\text{nFP, free}} \quad (4)$$

Where  $D_{\text{HOD}}$  is the diffusion coefficient of the solvent at the given sunset yellow concentration and  $D_{\text{HOD}}^0$  is the figure extrapolated to infinite dilution.<sup>28</sup> Eq 3 can then be solved for the associated fraction of the small molecule probe:

$$\chi_{\text{asc}} = \frac{D_{\text{nFP}} - D_{\text{nFP, free}}^{\text{corr}}}{D_{\text{SSY}} - D_{\text{nFP, free}}^{\text{corr}}} \quad (5)$$

### *Chemical shift analysis*

The variation in  $^1\text{H}$  chemical shift as a function of sunset yellow concentration was modelled using an isodesmic model of indefinite association.<sup>28,29,31</sup> Briefly, this model describes the observed chemical shift as the weighted average of three contributing chemical shifts: that of the monomer, molecules at the ends of the stacks and those within the aggregates. This assumes only nearest neighbour interactions contribute significantly to the association-induced change in chemical shift. Assuming isodesmic behaviour and that molecules at the ends of the stack receive half the shielding of those in the interior, then the observed chemical shift can be expressed as:<sup>31</sup>

$$\delta_{\text{obs}} = \delta_{\text{mon}} + (\delta_{\text{int}} - \delta_{\text{mon}}) \frac{2K_{\text{eq}}c_{\text{T}} + 1 - \sqrt{4K_{\text{eq}}c_{\text{T}} + 1}}{2K_{\text{eq}}c_{\text{T}}} \quad (6)$$

Where  $K_{\text{eq}}$  is the equilibrium constant for addition of a monomer to the aggregate,  $c_{\text{T}}$  is the total concentration and  $\delta_{\text{obs}}$ ,  $\delta_{\text{mon}}$  and  $\delta_{\text{int}}$  are the observed chemical shifts and those of the monomer and interior species respectively.

The isodesmic model can be extended to account for the interaction of a second species with aggregates.<sup>31</sup> The usual assumption employed here is that the second species is present at low concentration. This model requires two additional equilibrium constants, the first  $K_{\text{end}}$  describes the binding of the second species to the end of an aggregate, while the second  $K_{\text{int}}$  relates to the incorporation of the second

species into the interior of the aggregate. The observed chemical shift of the second species, denoted  $B$  is given by:

$$\delta_{\text{obs}}^B = a\delta_{\text{mon}}^B + b\delta_{\text{end}}^B + c\delta_{\text{int}}^B \quad (7)$$

where the mole fractions  $a$ ,  $b$  and  $c$  are given as:

$$\begin{aligned} a &= \frac{1}{1 + K_{\text{end}}[A_e] + K_{\text{end}}K_{\text{int}}[A_e]^2} \\ b &= \frac{K_{\text{end}}[A_e]}{1 + K_{\text{end}}[A_e] + K_{\text{end}}K_{\text{int}}[A_e]^2} \\ c &= \frac{K_{\text{end}}K_{\text{int}}[A_e]^2}{1 + K_{\text{end}}[A_e] + K_{\text{end}}K_{\text{int}}[A_e]^2} \end{aligned} \quad (8)$$

With the concentration of stack ends being:

$$[A_e] = 2c_T(1 - \alpha K_{\text{eq}}c_T) \quad (9)$$

$K_{\text{eq}}$  and  $c_T$  are the same as in eq (6). These models were implemented using the open source SciPy modules<sup>35</sup> of the python programming language, as reported previously.<sup>28,29</sup>

## Results and Discussion

### *Diffusion measurements*

Previous investigations have provided an insight into the aggregation behaviour of sunset yellow using diffusion NMR measurements.<sup>18,28,29</sup> When small molecule probes have been used, e.g. fluorophenol,<sup>28</sup> the relative proportion was kept small (1 mol%) so as to limit any possible disruption to the aggregates. In this study, the amount of fluorophenol probe is increased from this base level up to equimolar amounts of the probe and sunset yellow. Diffusion NMR measurements were recorded for a series of samples containing between 1 and 100 mol% 3-fluorophenol, across a range of sunset yellow concentrations (50 to 400 mM), monitoring both the

<sup>1</sup>H signals of sunset yellow and the <sup>19</sup>F signals of the 3-fluorophenol. These data are plotted as surface plots in Figure 2(a) and (b) respectively. As the concentration of sunset yellow is increased there is a monotonic decrease in the observed <sup>1</sup>H diffusion coefficient of the sunset yellow as larger scale aggregates become the predominant species in solution as observed previously.<sup>18,28</sup> What is notable, however, is that increasing the relative proportion of 3-fluorophenol from 1 mol % to 100 mol% has very little influence on the trend in the <sup>1</sup>H diffusion coefficient. As larger relative amounts of 3-fluorophenol are used, the same trend in sunset yellow diffusion coefficient is observed, suggesting that the 3-fluorophenol probe is having limited influence on the macroscopic assembly state of the sunset yellow, at least on the time scale of the diffusion labelling period  $\Delta$ .<sup>34</sup> This is slightly unexpected since there is evidence for an interaction between fluorophenol and sunset yellow,<sup>28</sup> it might be anticipated that increasing the concentration of the probe to equimolar levels would potentially result in a disruption, or at least modification, of the assembly state of sunset yellow, since there are some differences in the size and structure of the two species.<sup>36</sup>

At low relative concentrations, the <sup>1</sup>H signals of the 3-fluorophenol are too low in signal to noise to enable the reliable determination of the diffusion coefficients against the background of much more intense sunset yellow signals. Therefore the behaviour of the small molecule probe is followed by its unique NMR-handle, the <sup>19</sup>F nucleus. Figure 2(b) shows the <sup>19</sup>F-observed diffusion coefficients of the 3-fluorophenol recorded on the same samples as in Figure 2(a). These data broadly follow the same trends as for the diffusion coefficients of the sunset yellow itself, except that the diffusion coefficient is around a factor of two larger, indicating that the

3-fluorophenol is neither free, nor completely associated with the sunset yellow aggregates.<sup>28,29</sup> If there were complete association, then it should have the same measured diffusion coefficient as the sunset yellow aggregates, regardless of the nucleus used for the measurement. The 3-fluorophenol diffusion is slower than that observed in the absence of sunset yellow at the same equivalent concentration (data not shown).<sup>28</sup> If there were no association between the sunset yellow and the 3-fluorophenol, then a faster diffusion coefficient, very close to that of the free fluorophenol, would be expected. Also, there is no evidence for the self-assembly of fluorophenol in aqueous solution as the  $^{19}\text{F}$  diffusion coefficient remains unchanged with increasing concentration.<sup>28</sup> Interestingly, this is not the case in the structurally similar fluoronaphthoic acids.<sup>30</sup> In addition, as a single  $^{19}\text{F}$  resonance is observed, the exchange of the probe between the free and associated states is therefore rapid on both the NMR and diffusion labelling time scales. These observations together have been interpreted as showing a weak interaction between the fluorophenol probe and the sunset yellow.<sup>28,29</sup>

Following the approach used previously<sup>28,29</sup> the measured diffusion coefficients for the sunset yellow and 3-fluorophenol can be used to determine a bound fraction  $\chi_{\text{asc}}$ , i.e. the percentage of 3-fluorophenol associated with the sunset yellow aggregates. This fraction is determined via the Lindman law, using eq 5, and shown as a surface plot in Figure 3(a). The diffusion coefficient of the solvent,  $D_{\text{HOD}}$ , as a function of sample composition is shown in Figure S1 (Supplementary Information). The trend in the bound fraction is similar to that observed previously,<sup>28</sup> in that there is a significant increase in bound fraction with increasing sunset yellow concentration. However, as the relative amount of 3-fluorophenol is increased, there is now a very slight decrease

in bound fraction of probe molecule at higher sunset yellow concentrations. Given the large distribution of SSY aggregates present in solution, and the small equilibrium constants for binding of the small molecules to sunset yellow (see below), then it is perhaps unsurprising that the bound fraction has only a limited dependence on the amount of fluorophenol within the samples. Figure 3(b) replots the same data in terms of the absolute concentration of bound 3-fluorophenol, rather than the bound fraction. This alternative view shows that while the bound fraction may decrease slightly with increasing relative amount of 3-fluorophenol, the absolute amount of bound probe does increase as would be expected given the large number of potential binding sites across the aggregate distribution.

The plot of bound fraction as a function of sunset yellow concentration (Figure 3(a)) shows two regimes, above and below 100 mM. In conjunction with chemical shift data below this has previously been interpreted as a change in the binding mode of the probe molecule from predominately at the end of the assemblies to an intercalation-like mode in the interior of the stacks.<sup>28,29</sup> The behaviour of the bound fraction as a function of concentration can be modelled empirically as a biexponential function of concentration.<sup>28</sup> The amplitudes of the two exponential components are plotted as a function of 3-fluorophenol concentration in Figure 3(c). It should be noted that as the 400 mM sunset yellow sample was only investigated with 3-fluorophenol amounts up to 50 mol% due to solubilities issues, there is only a single  $\chi_{\text{asc}}$  data point above 100 mM sunset yellow (i.e. 200 mM) making a reliable determination of the second component of the biexponential fit difficult, hence the exponential amplitude analysis is given up to 50 mol% 3-fluorophenol. The data in Figure 3(c) shows that there is a gradual, but consistent variation in the balance between the two exponential

components from approximately 45:55 split at low concentrations of 3-fluorophenol (consistent with previous data<sup>28</sup>) to 40:60 at higher mole fractions. This suggests that as the amount of 3FP is increased a larger range of binding sites are being occupied, with an associated change in binding mode, as observed previously for higher concentrations of sunset yellow.<sup>28,29</sup>

### *Chemical shift variation*

While the variation in diffusion coefficients give insights into bulk changes occurring in the samples as a function of both sunset yellow and fluorophenol concentration, NMR spectroscopy can also give more detailed, i.e. atomic, information via changes in observed chemical shifts. Increases in shielding due to the  $\pi$ - $\pi$  stacking interactions means the observed chemical shift is an excellent reporter of aggregation state,<sup>16,37</sup> and provides a measure of the monomer-aggregate equilibrium.<sup>31</sup> Figure 4(a) shows the <sup>1</sup>H chemical shift of the H1 proton on sunset yellow as a function of both sunset yellow concentration and amount of added 3-fluorophenol. This proton is representative of the chemical shift changes observed across the whole of the sunset yellow proton sites.<sup>28</sup> From these data it is clear that there is an increase in shielding (i.e. a decrease in the observed chemical shift) as the concentration of sunset yellow is increased, consistent with an increase in  $\pi$ - $\pi$  stacking as larger aggregates are formed. Additionally, there is little change in the <sup>1</sup>H chemical shift as a function of added 3-fluorophenol, except towards the highest relative concentrations (100 mol%), indicating that the 3FP is causing limited disruption of the assemblies. Using chemical shift data across all proton sites in sunset yellow and globally fitting these concentration-dependent changes to the isodesmic model given in eq 6 yields equilibrium constants for the self-association. Results for each 3-fluorophenol

concentration are plotted in Figure 4(b). These equilibrium constants show that there is a small, but clear decrease in the stability of the sunset yellow aggregates as the relative amount of 3-fluorophenol is increased. The presence of a small amount (1 mol%) of any of the fluorophenol isomers has previously been shown to cause a small increase in stability of sunset yellow aggregates,<sup>28</sup> however, clearly larger amounts of the small molecule probe are likely to cause increased disruption as greater numbers of probe molecule interact with the assemblies. Strictly, the isodesmic model assumes that there is a single species undergoing self-association and that the equilibrium constants are equal for addition of each subsequent monomer.<sup>31</sup> There is some evidence that the isodesmic model does not offer a complete description of the association occurring in sunset yellow.<sup>25,38</sup> In this case, the use of this model enables general trends to be determined using a simple model and affords comparison with our previous analyses.<sup>28–30</sup>

The fluorine-19 site of the fluorophenol provides a key reporter on the environment experienced by this small molecule probe during its interaction with the sunset yellow and hence suggest possible binding modes.<sup>28,29</sup> Figure 5(a) shows the fluorine-19 chemical shift of 3-fluorophenol plotted as a function of both sunset yellow concentration and relative amount of fluorophenol probe added (similar to Figure 4(a)). As noted previously,<sup>28–30</sup> the <sup>19</sup>F chemical shift shows a clear biphasic trend with an initial decrease in chemical shift for sunset yellow concentrations below around 100 mM, followed by an increase (i.e. trend to less negative chemical shifts) to higher concentrations. This has been interpreted as a change in the binding mode of the 3-fluorophenol probe molecule from predominately end-on to the aggregates to insertion into the aggregates themselves.<sup>28</sup> As commented on above, regarding the



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3 sunset yellow  $^1\text{H}$  chemical shifts, there is little variation observed in the 3-  
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5 fluorophenol  $^{19}\text{F}$  chemical shifts with increasing probe molecule concentration, except  
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7 at the highest sunset yellow concentrations in which case there is a small decreasing  
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9 (i.e. more negative) change.  
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14 The isodesmic model can be modified to include the interaction of a second molecule  
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16 with a larger assembly.<sup>28,29,31</sup> The model given in eqs 7-9 comprises two additional  
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18 equilibrium constants describing two interaction modes of the second molecule: end-  
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20 on ( $K_{\text{end}}$ ) and insertion into the aggregate stack ( $K_{\text{int}}$ ). Global fitting this model to the  
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22  $^{19}\text{F}$  chemical shift data as a function of sunset yellow concentration in Figure 5(a), at  
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24 each 3-fluorophenol concentration, results in the equilibrium constants shown in  
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26 Figure 5(b). The equilibrium constants are broadly unaffected by the increasing  
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28 relative proportion of 3-fluorophenol, however, there is a very slight decrease in  $K_{\text{end}}$ ,  
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30 suggesting that at higher 3-fluorophenol concentrations, there is maybe a slight shift  
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32 to greater incorporation of 3FP into the sunset yellow stacks. Taken together, the  
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34 variation in both the  $^1\text{H}$  and  $^{19}\text{F}$  chemical shifts of sunset yellow and 3-fluorophenol  
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36 respectively, suggests that there is a certain degree of interaction between the probe  
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38 and the sunset yellow aggregates which occurs from low relative concentrations  
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40 (1mol%) with very little change in the nature of these interactions as the mole fraction  
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42 of the probe molecule is increased. There is little evidence of significant  
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44 destabilisation of the sunset yellow aggregates induced by the addition of larger  
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46 quantities 3-fluorophenol.  
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56 *Influence of fluorophenol isomerism*  
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So far, the results of using 3-fluorophenol to demonstrate the interaction of a small molecule probe with sunset yellow have been presented. As with previous work,<sup>28</sup> it is interesting to explore the role of structural isomerism in the small molecule via the comparison of 2-, 3- and 4-fluorophenols. Rather than repeat wholesale the results presented above, three specific sunset yellow concentrations were chosen so as to sample three regions of the sunset yellow concentration space, i.e. below (40 mM), at (100 mM) and above (250 mM) the change in fluorophenol binding mode reported previously.<sup>28,29</sup> Both diffusion coefficient and chemical shift data (<sup>1</sup>H for SSY and <sup>19</sup>F for fluorophenol) are shown in Figure 6. It is immediately clear from these data that the choice of fluorophenol isomer has limited effect on the variation in diffusion coefficients of sunset yellow (<sup>1</sup>H-measured, Figure 6(a)) or fluorophenol (<sup>19</sup>F-measured, Figure 6(b)), the same trends which were observed in Figure 2 are apparent here also. Likewise, the trends in the chemical shifts (<sup>1</sup>H of SSY in Figure 6(c) and <sup>19</sup>F of nFP in Figure 6(d)) are largely unaffected by the choice of fluorophenol isomer.

Figure 7(a) shows the associated fraction of fluorophenol calculated according to eq 5 and as the total concentration of bound fluorophenol in Figure 7(b). In both of these plots, there is little variation among the three isomers, except that 3-fluorophenol shows slightly lower total association at the higher sunset yellow concentration used. In the case of some isomers of (fluoro)naphthoic acids interacting with sunset yellow the polarity of the small molecule was seen to have a small effect on the association with the aggregates.<sup>30</sup> For the fluorophenol isomers, there is only a small change in the dipole moment (Table 1), especially for the 3- and 4-fluoro isomers. If polarity were a major contributing factor then the 2-fluorophenol isomer should show significant deviation compared with the other isomers.

## Conclusions

Sunset yellow is often used as an archetypical example of a small molecule which undergoes  $\pi$ - $\pi$  stacking in aqueous solution. Previously, we have investigated the interaction of sunset yellow with other small molecules at low relative concentration.<sup>28–30</sup> Here, we have established that for the three fluorophenol isomers increasing the relative concentration from 1 mol% to equimolar results in little change in the sunset yellow aggregation behaviour beyond that induced by the initial addition of fluorophenol. At high concentrations of both sunset yellow and fluorophenol, a maximum of around 40% of the available fluorophenol is bound to the sunset yellow aggregations across all relative concentrations of small molecule probe. This implies that the binding sites on the sunset yellow are saturated, hence increasing the fluorophenol just adds to the pool of unbound molecules, and causes no further disruption to the association of the sunset yellow. The lack of disruption on addition of the fluorophenol is attributed to the close structural similarities shared with the sunset yellow and its small size. In the case of fluorophenol there is, therefore, little cause for concern with the amount of small molecule probe used to investigate the aggregation state of the sunset yellow. However, this is unlikely to be true in general, for a given probe and aggregating species, particularly, dyes and other species used to probe protein aggregation which have been shown to cause disruption.<sup>4,39</sup>

## Supporting Information Available

HOD diffusion data, alternative views of the data presented in Figures 2, 3, 4 and 5.

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Tables and Table Captions

Table 1: Dipole moments for the three fluorophenol isomers, calculated using Avogadro.<sup>40</sup>

Species	Dipole Moment (D)
2-fluorophenol	3.947
3-fluorophenol	3.061
4-fluorophenol	3.026

Figure Captions

Figure 1: Structures of the compounds used. Sunset yellow **1** is shown in the more favoured hydrazone tautomer.<sup>3,13</sup>

Figure 2: (a) <sup>1</sup>H-measured diffusion coefficients for sunset yellow as a function of concentration and relative amount of added 3-fluorophenol. (b) <sup>19</sup>F-measured diffusion coefficients of the 3-fluorophenol component of the same samples as in (a). 2D representations of the surface plots are presented as Figure S2 in Supplementary Information.

Figure 3: (a) shows the associated fraction of 3-fluorophenol as a surface plot depending on the sunset yellow and relative 3-fluorophenol concentrations. 2D representations of the surface plot is presented as Figure S3 in Supplementary Information. (b) is the same data scaled to show the molar concentration of bound 3-fluorophenol. (c) shows the amplitudes of the two components in a biexponential fit

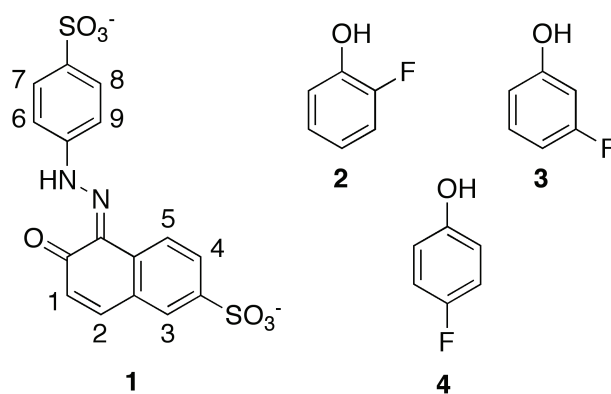
of slices of the data presented in (a) taken at constant relative concentration of 3-fluorophenol.

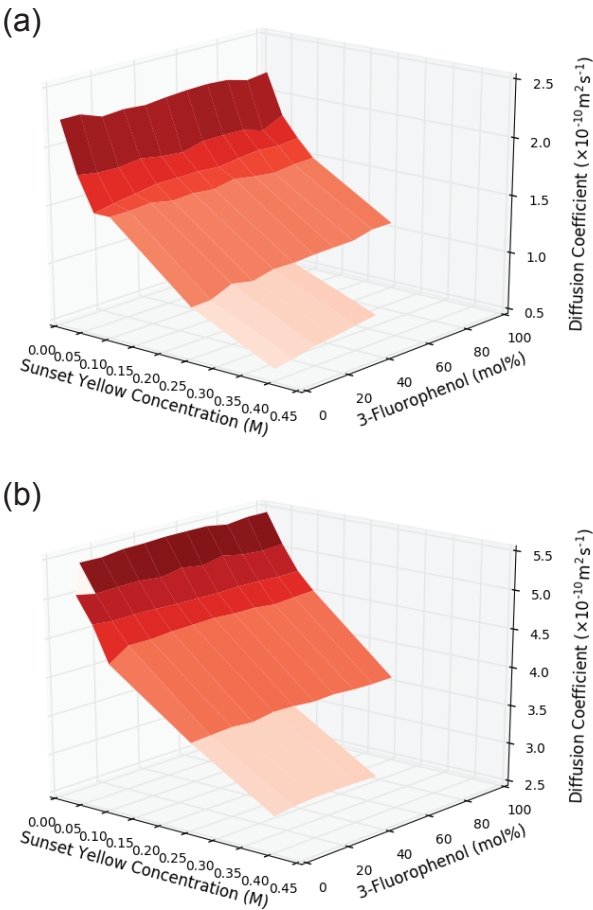
*Figure 4:* (a)  $^1\text{H}$  chemical shift of proton H1 of sunset yellow as a surface plot depending on the sunset yellow and relative 3-fluorophenol concentrations. 2D representations of the surface plot is presented as Figure S4 in Supplementary Information. (b) Equilibrium constant for the association of sunset yellow calculated using the isodesmic model (eq 6) as a function of 3-fluorophenol concentration.

*Figure 5:* (a)  $^{19}\text{F}$  chemical shift of 3-fluorophenol as a surface plot depending on the sunset yellow and relative 3-fluorophenol concentrations. 2D representations of the surface plot is presented as Figure S5 in Supplementary Information. (b) Equilibrium constants for the interaction of a second molecule with an aggregate of sunset yellow calculated using the modified isodesmic model (eqs 7-9) as a function of 3-fluorophenol concentration.

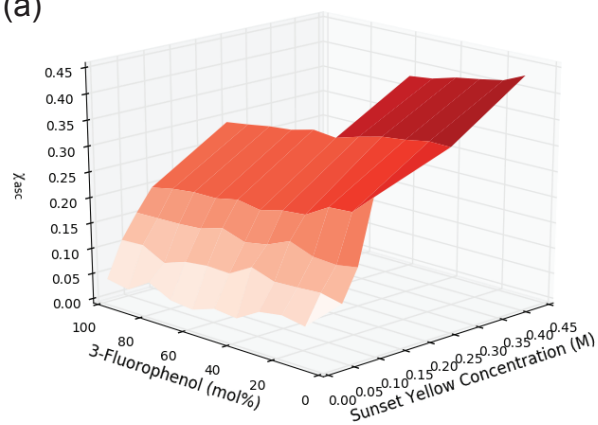
*Figure 6:* (a)  $^1\text{H}$ -measured diffusion coefficients for SSY as a function of the fluorophenol concentration for the three isomers. (b)  $^{19}\text{F}$ -measured diffusion coefficients for the same samples as in (a). (c) and (d) show the  $^1\text{H}$  and  $^{19}\text{F}$  chemical shifts for SSY and fluorophenol. In (d), the upper, middle and lower traces correspond to 2-fluorophenol, 3-fluorophenol and 4-fluorophenol respectively. The filled, shaded and open symbols refer to the 40, 100 and 250 mM sunset yellow samples, using the same colour scheme as the other panels.

*Figure 7:* (a) bound fraction of fluorophenol as a function of relative fluorophenol concentration determined using eq 5 for each of the isomers of fluorophenol. (b) shows the same data, but this time plotted as the absolute concentration of bound fluorophenol. The colour scheme is the same as that in Figure 6.

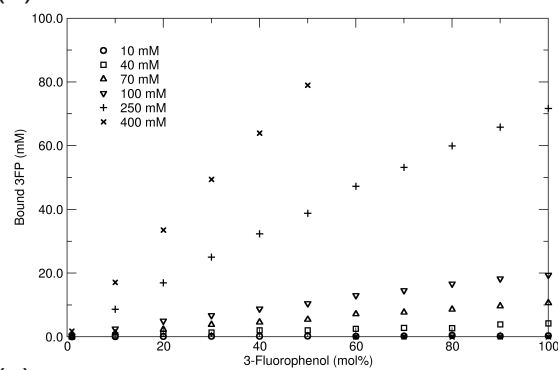




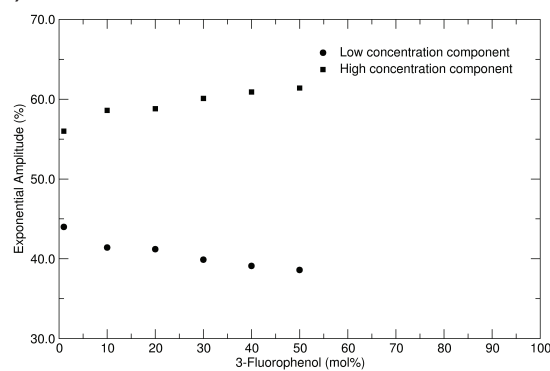
(a)

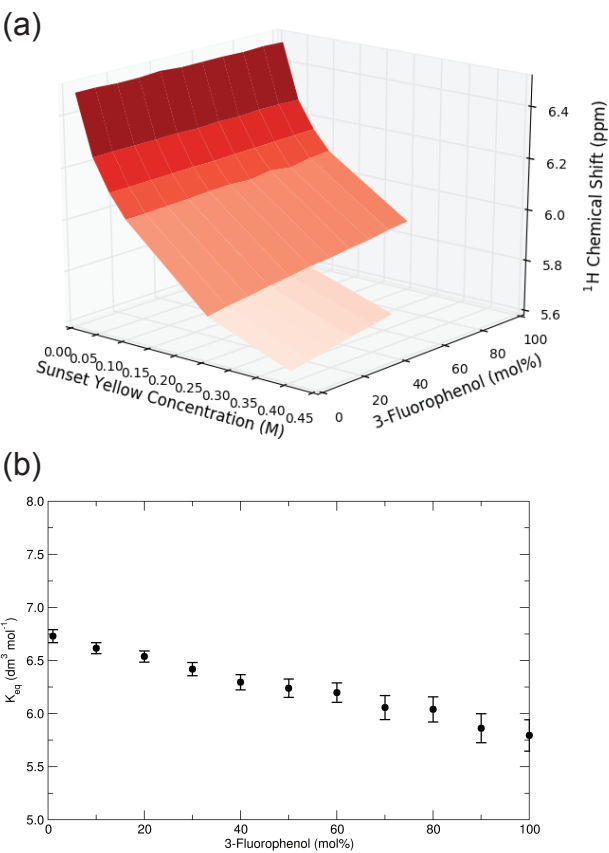


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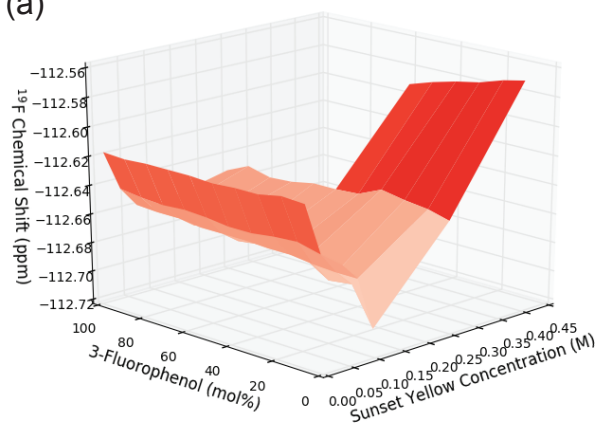


(c)

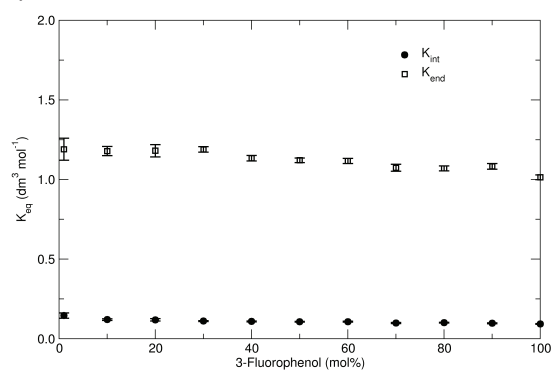




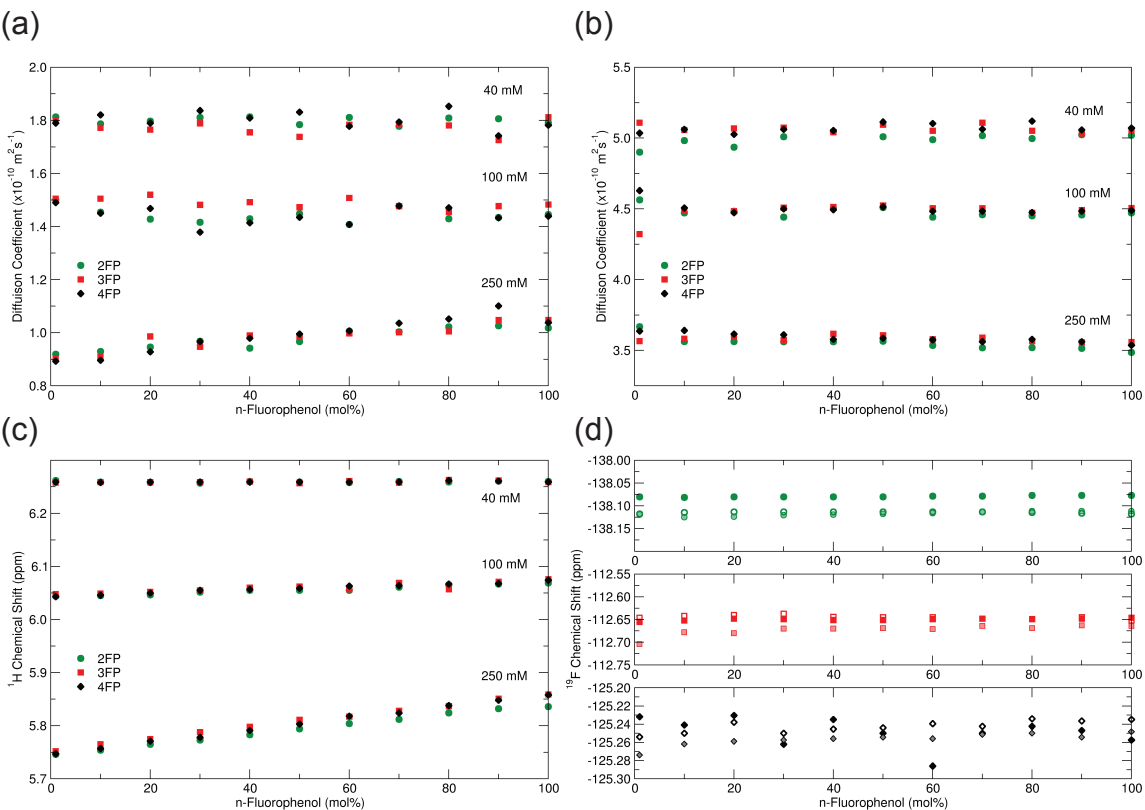
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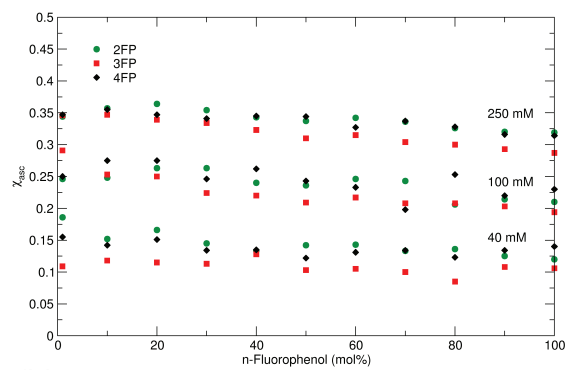
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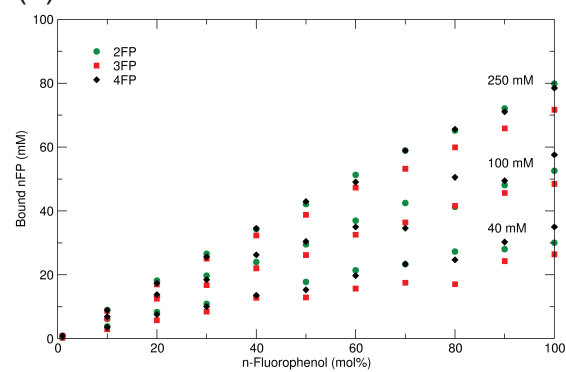


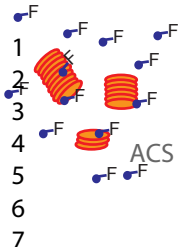


(a)



(b)





Diffusion



Chemical

shifts

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